Human Embryonic Stem Cell-Derived Epithelial Cells in a Novel *In Vitro* Model of Vocal Mucosa

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A satisfactory *in vitro* model of vocal fold mucosa does not exist, thus precluding a systematic, controlled study of vocal fold biology and biomechanics. We sought to create a valid, reproducible three-dimensional (3D) *in vitro* model of human origin of vocal fold mucosa of human origin. We hypothesized that coculture of human embryonic stem cell (hESC)-derived simple epithelial cells with primary vocal fold fibroblasts under appropriate conditions would elicit morphogenesis of progenitor cells into vocal fold epithelial-like cells and creation of a basement membrane. Using an *in vitro* prospective study design, hESCs were differentiated into cells that coexpressed the simple epithelial cell marker, keratin 18 (K18), and the transcription factor, p63. These simple epithelial cells were cocultured with primary vocal fold fibroblasts seeded in a collagen gel scaffold. The cells were cultured for 3 weeks in a keratinocyte medium at an air–liquid interface. After that time, the engineered mucosa demonstrated a stratified, squamous epithelium and a continuous basement membrane recapitulating the key morphologic and phenotypic characteristics of native vocal fold mucosa. hESC-derived epithelial cells exhibited positive staining for vocal fold stratified, squamous epithelial markers, keratin 13 (K13) and 14 (K14), as well as tight junctions, adherens junctions, gap junctions, and desmosomes. Despite the presence of components critical for epithelial structural integrity, the epithelium demonstrated greater permeability than native tissue indicating compromised functional integrity. While further work is warranted to improve functional barrier integrity, this study demonstrates that hESC-derived epithelial progenitor cells can be engineered to create a replicable 3D *in vitro* model of vocal fold mucosa featuring a multilayered, terminally differentiated epithelium.

Introduction

VOCAL FOLD DISORDERS are associated with substantial and far-ranging economic,¹ social, and psychological costs.² Behavioral, medical, and surgical techniques are routinely used to manage vocal fold disorders; however, suboptimal patient outcomes are reported. For example, more than one third of patients who undergo vocal fold surgery report impaired vocal fold function after intervention.³ A better understanding of the pathophysiology of diseases in the vocal fold will likely lead to improved treatment outcomes. Systematic *in vivo* study of vocal fold disorders is challenging as the vocal folds are housed deep within the larynx and are vulnerable to manipulation. Creation of an engineered vocal fold tissue construct offers a promising approach to studying vocal fold biology and biomechanics in health and disease.

There is currently no satisfactory *in vitro* model of vocal folds. Our goal was to develop a model that mirrors the cell

composition and three-dimensional (3D) environment of vocal fold mucosa. Epithelial cells and fibroblasts likely play a significant role in vocal fold biology; however, the cellular and molecular mechanisms underlying their interactions in health, injury, and disease have been largely unexplored. The primary reason for this is that vocal fold epithelial cell lines do not exist. Compounding this problem, epithelial cells are difficult to study and cannot be removed from the healthy larynx without engendering a significant and unacceptable risk to vocal fold function. Therefore, a valid, reproducible model of vocal fold mucosa populated by a continuous, large-scale supply of stable epithelial cells that demonstrate the key morphologic, genotypic, and phenotypic similarities of native vocal fold epithelial cells is needed. The model will serve as an invaluable tool for examining and manipulating cell signal pathways in development, health, and injury; modeling vocal fold damage and disease; and testing safety and effectiveness of novel cytoprotective and regenerative treatments *in vitro*. In the present study, we sought to

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develop a physiologically relevant, 3D *in vitro* vocal fold model of human origin derived from embryonic stem cells (ESCs).

While both monolayer and 3D cultures are used in cell biology and tissue-engineering studies, 3D models create a cell culture system that better approximates the *in vivo* environment. In a previous study of vocal fold fibroblasts in monolayer and 3D cultures,⁴ we found that fibroblasts cultured in the 3D system demonstrated phenotypes that are more consistent with vocal fold tissue than those grown in monolayers on the same substrate. Two types of 3D vocal fold mucosa culture models have been proposed previously. Yamaguchi *et al.*⁵ developed an organotypic model of vocal fold mucosa using primary porcine vocal fold fibroblasts and epithelial cells. In their model, epithelial cells obtained at first passage were placed on a collagen I gel seeded with fibroblasts. Once the epithelial layer reached confluence, the cells were cultured at the air-liquid interface (ALI). The final construct demonstrated multilayered, differentiated epithelial cell layers consistent with the native epithelium. However, the model lacked a critical element, a complete basement membrane. The basement membrane is an important component of the mucosa: it provides a point of attachment for epithelial cells to the underlying extracellular matrix, forms a mechanical barrier between the environment and underlying tissue, and plays a critical role in growth factor cell signaling⁶ in pathogenesis and repair, cell differentiation,⁷ and tissue development.⁸ Moreover, while this model demonstrated important features of vocal fold mucosa *in vitro*, given the poor accessibility, limited proliferative capacity, and high level of heterogeneity of primary vocal fold epithelial cells, as well as the absence of primary or transformed epithelial cell lines, it would be challenging to create reproducible, robust organotypic models using this technique.

Stem cells offer a promising solution to the absence of epithelial cell lines for *in vitro* work. As they demonstrate the unique characteristics of pluripotency and self-renewal, in theory, they can provide large quantities of any cell type in the body. Long and colleagues⁹ created a tissue-engineered, bilayered, vocal fold mucosa populated by human adipose stem cells (ASCs). In their model, ASCs were seeded in a fibrin gel. In the presence of an ALI and epidermal growth factor (EGF), cells near the surface were positive for K8, a marker of a simple epithelium. Further, no basement membrane was shown. This model offers important advantages over the earlier model, including the use of readily available adult stem cells and the potential for development of an epithelium. However, the lack of a confluent epithelial layer limits the model's applicability for *in vitro* study of vocal fold mucosa physiology.

A key function of the epithelium is to provide a physical, selective barrier between the external environment and underlying tissue. Therefore, in addition to demonstrating an epithelial phenotype, candidate tissue constructs must also demonstrate the presence of important components of the epithelial barrier, including intercellular junctions. The native vocal fold epithelium contains intercellular junctions, including tight junctions,^{10,11} gap junctions,¹² adherens junctions,¹¹ and desmosomes.¹⁰ While Long and colleagues⁹ reported the presence of e-cadherin, a marker of adherens junctions, in their model, further evidence of intercellular junctions was not provided. The presence of intercellular

junctions in a model of vocal fold mucosa is important as these structural features render the native epithelium tight as evidenced by transepithelial resistance in animal models in excess of 300 ohms.^{10,13} No model to date has demonstrated functional barrier integrity.

The purpose of our study was to develop a reproducible, robust 3D model of vocal fold mucosa that demonstrates key features of mucosa, including stratified, differentiated squamous epithelial cells, as well as a complete and continuous basement membrane on a gel scaffold embedded with fibroblasts. Palecek and colleagues previously developed a reproducible, valid organotypic skin culture model that featured a stratified, differentiated epithelium. The epidermal construct was generated from keratinocyte progenitor epithelial cells differentiated from human ESCs (hESCs) by retinoic acid and bone morphogenetic protein signaling.¹⁴ To elicit epithelial morphogenesis, the progenitor cells were derived from these simple epithelial cells and seeded on a dermal construct composed of the collagen I gel and human dermal fibroblasts and grown at an ALI.¹⁵ The method yielded an organotypic culture that recapitulated many features of skin, including a cornified, stratified epithelium. Based on those findings, we hypothesized that coculture of hESC-derived simple epithelial cells with vocal fold fibroblasts on a collagen raft would elicit morphogenesis of progenitor cells into vocal fold epithelial-like cells.

Materials and Methods

Derivation of simple epithelial cells

Epithelial progenitor cells were differentiated from a hESC line (WA09; passage 20–26) as described by Metallo *et al.*¹⁴ Briefly, hESCs were cultured on irradiated mouse embryonic fibroblasts, then plated on Matrigel-coated plates, and cultured in mTeSR1 (StemCell Technologies, Vancouver, CA) for hESC expansion. For differentiation, hESCs were cultured on Matrigel (BD Biosciences, San Jose, CA) in unconditioned hESC medium (UM) supplemented with retinoic acid (UM + RA): the DMEM/F12 containing 20% knockout serum replacer (Life Technologies Corporation, Carlsbad, CA), 1×MEM nonessential amino acids, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 1 μM all-trans retinoic acid (Sigma Aldrich, St. Louis, MI). Cells were cultured in UM + RA for 7 days with the medium changed daily.

Characterization of simple epithelial cells

hESC-derived epithelial cells were characterized using standard polymerase chain reaction (PCR), flow cytometry, and immunocytochemistry before and following 1 week of treatment with retinoic acid. For PCR, the hESCs were dissociated with 0.25% Trypsin-EDTA (Life Technologies Corporation) and lysed in preparation for RNA extraction. Total RNA was extracted from using the RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Corresponding cDNA was made from 1 μg of RNA using Omniscript reverse transcriptase (Qiagen) and oligo-dT primers (Life Technologies Corporation). RT-PCR was performed using 1 μg of cDNA per reaction with GoTaq polymerase (Promega, Madison, WI) and gene-specific primers (Table 1). Products were run on a 2% agarose gel and imaged after incubation in the ethidium bromide solution.

TABLE 1. PRIMER SEQUENCES FOR REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Gene	Forward primer	Reverse primer	Size (bp)
Oct4	AGTTTGTGCCAGGGTTTTTG	ACTTCACCTTCCCTCCAACC	113
K18	CCGTCTTGCTGCTGATGACT	GGCCTTTTACTTCCTCTTCGTG	200
K13	CTGGATGAGCTCACTCTGTCTAAG	CTTCATCTCCTCTTCATGGTTCTT	102
p63	ACCAGAGATGGGCAAGTCCT	ATCCGCCTTCCTGTCTCTTC	80
GAPDH	CACCGTCAAGGCTGAGAACG	GCCCCACTTGATTTTGGAGG	91

K13, keratin 13; K18, keratin 18.

For flow cytometry, cells were incubated with the following human-specific primary antibodies 1 week after treatment with retinoic acid: Nanog, a marker of undifferentiated stem cells (1:50; BD Biosciences); K18, a marker of simple epithelium and found in preimplantation embryos¹⁶ (1:100; Abcore, Ramona, CA), keratin 13 (K13; 1:100; Abcam, Cambridge, MA) and keratin 14 (K14; 1:100; Thermo Scientific, Kalamazoo, MI), markers of the stratified squamous epithelium; and the transcription factor p63, a basal epithelial cell marker necessary for development of all stratified epithelial tissue¹⁷ (1: 25; Santa Cruz Biotechnology, Santa Cruz, CA). Species-specific secondary antibodies were obtained from Life Technologies Corporation and were conjugated to Alexa 488 or 594 dyes. Cells stained with the secondary antibodies only served as negative controls. Results are expressed as mean values $\pm$ standard error of percentages of positive staining for each antibody. Cells were analyzed on a FACSCalibur using CellQuest software or a BD LSRII flow cytometer using BD FACSDiva software (BD Biosciences).

hESC-derived simple epithelial cells were further characterized by standard immunofluorescence using antibodies directed against the transcription factor p63 (1:25; Santa Cruz Biotechnology), Nanog (1:800; Cell Signaling Technology, Danvers, MA), K18 and K14, and isotype controls. Cells in monolayer were fixed in 4% paraformaldehyde for 20 min, rinsed in phosphate-buffered saline (PBS), and incubated in 5% milk with 0.4% (v/v) Triton X-100 in PBS for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C in the blocking buffer and, after subsequent washes in PBS, species-specific fluorophore-conjugated secondary antibodies (Life Technologies Corporation) were applied in the dark for 1 h at room temperature. The Hoechst dye provided nuclear staining.

Creation of a 3D tissue construct

The scaffold consisted of a collagen I gel seeded with fibroblasts. It served to mimic the collagen-rich vocal fold

lamina propria. Scaffolding was prepared as described elsewhere.¹⁵ Collagen I gel (final concentration of 4 mg/mL; BD Biosciences) was mixed with ice-cold 10 $\times$ DMEM and 10% fetal bovine serum (FBS) (Sigma Aldrich) containing human primary vocal fold fibroblasts at a density of 5 $\times$ 10⁵ cells per mL of the final volume of gel. The human vocal fold fibroblast cell line, 21T (passage 5–6), was used in this study. This primary cell line was developed from pathogen-free vocal folds obtained from a 21-year-old male donor and has been characterized previously.¹⁸ The protocol was approved by the Institutional Review Board of the University of Wisconsin–Madison.

Two milliliters of gel was added to each six-well insert. The gels were allowed to set in an incubator (37°C, 5% CO₂/95% O₂) for 1 h, and then flooded with flavinoid adenine dinucleotide (FAD) media. The media were composed of Ham's F-12/DMEM (3:1 ratio), FBS (2.5%), hydrocortisone (0.4 μ g/mL), cholera toxin (8.4 ng/mL), insulin (5 μ g/mL), adenine (24 μ g/mL), EGF (10 ng/mL), penicillin (100 U/mL), and streptomycin (0.01 mg/mL). A defined serum-free keratinocyte medium (DSFM; Invitrogen, Carlsbad, CA) with and without additional calcium (1 mM) was used in lieu of FAD in some gels. However, use of the DSFM was discontinued because the medium yielded inferior results.

The K18+/p63+ simple epithelial cells were seeded on collagen I gels at a density of approximately three million cells suspended in 100 μ L of FAD per six-well insert. The cells were allowed to attach to the gels for 2 h after which, the gels were flooded with FAD and returned to the incubator. An ALI was created 2 days later by removing media from the gel surface (Fig. 1). Cultures were grown at the ALI for 19–21 days. Experiments were performed in triplicate.

Characterization of tissue constructs

Vocal fold mucosa cultures were prepared for histological and immunohistochemical analyses using standard procedures. Organotypic cultures were fixed in 10% neutral

FIG. 1. Schematic of coculture of keratin 18+(K18+)/p63+-derived epithelial cells and human primary vocal fold fibroblasts. Fibroblasts were seeded in a collagen I gel and left to contract for 2 days (A). Derived epithelial cells were seeded on the gel and submerged in media for 2 days (B). Media were removed from the construct surface and cells were cultured at the air-liquid interface for 3 weeks (C).

FIG. 2. Flow cytometry revealed positive staining for K18 (A) and p63 (D) in derived cells, but not in cells treated with the secondary antibody only. Few cells (<3%) stained for K14 (B), K13 (E), and Nanog (C). Color images available online at www.liebertpub.com/tea

phosphate-buffered formalin (pH of 7.0) at room temperature and embedded in histogel (Thermo Scientific) to maintain structure. Cultures were processed overnight and embedded in paraffin. Five-micron coronal sections were obtained from each culture. Sections were prepared for hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC). H&E staining was completed using standard methods to examine construct morphology. IHC was performed using primary antibodies against the stratified squamous epithelium markers K14 (1:100; basal layer cells) and K13 (1 to 100; suprabasal layer cells); an intercellular junction marker e-cadherin (1 to 500; adherens junctions); and a basement membrane marker, laminin 5 (1 to 50). All antibodies were purchased from Abcam except for K14, which was purchased from Thermo Scientific. Antigen retrieval using sodium citrate and heat was performed and antibodies were applied overnight at 4°C. Labeling was detected using horseradish peroxidase and visualized with diaminobenzidine as the chromagen before counter staining with hematoxylin and mounting. Sections were viewed with a Nikon E600 microscope (Nikon, Melville, NY) and photographed using an Olympus DP71 microscope digital camera (Tokyo, Japan).

Electron microscopy

For standard transmission electron microscopy, gels were immersion fixed in 2.5% glutaraldehyde and 2%

FIG. 3. Polymerase chain reaction before (day 0) and after (day 7) derivation of simple epithelial cells. Strong Oct 4 expression was observed at day 0. K18+ /p63+ expression was noted at day 7 only. No K13 expression was found at either time points. The reference gene (GAPDH) was positive at both time points.

FIG. 4. Immunofluorescence confirmed that the derived epithelial cells stained positive for K18 (**A**), p63 (**B**) and K18 and p63 (**C**) (50- μ m scale bar). Color images available online at www.liebertpub.com/tea

FIG. 5. No staining was observed for K13 (**A**), K14 (**B**), and Nanog (**C**; 50- μ m scale bar for all images) in stem cell-derived simple epithelial cells. Hoechst dye was used as a nuclear stain. Color images available online at www.liebertpub.com/tea

FIG. 6. Hematoxylin and eosin (H&E) staining of a representative engineered mucosa showed a multilayered, non-cornified, squamous epithelium consistent with the vocal fold epithelium (200- μ m scale bar). Color images available online at www.liebertpub.com/tea

paraformaldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C. Gels were processed using routine techniques. Briefly, gels were washed in a 0.1 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature. Tissues were dehydrated in graded ethanol series, rinsed twice in propylene oxide, and embedded in Epon 812 epoxy resin (Polysciences, Inc., Warrington, PA) under vacuum. Finally, the samples were flat embedded between glass slides.

After resin polymerization, one of the two glass slides were removed and blank resin cylinders were glued to the sections. The gels were thin sectioned for TEM using a Leica EM UC6 Ultramicrotome and stained with Reynolds lead citrate and 8% uranyl acetate in 50% EtOH to increase contrast. The sections were viewed with a Philips CM120 electron microscope and images were captured with a MegaView III side mounted digital camera.

For permeability analysis, a heavy metal tracer, lanthanum nitrate (final concentration 1%; Electron Microscopy Sciences, Hatfield, PA) was added to the fixative and to reagents at each subsequent stage of processing, including buffer washes, osmium tetroxide, and alcohol rinses. Gels were sectioned and viewed as described above.

Results

We constructed a reproducible, *in vitro* model of vocal fold mucosa using simple epithelial cells derived from hESCs. The engineered model demonstrated key features of native mucosa, including the presence of a stratified, differentiated epithelium and a basement membrane.

Characterization of derived simple epithelial cells via flow cytometry before seeding on the gel showed a high level of homogenous expression of K18 ($97.6\% \pm 0.25\%$) and p63 ($94.9\% \pm 5.2\%$). Few cells expressed Nanog ($0.74\% \pm 0.49\%$), K14 ($2.12\% \pm 2.52\%$), and K13 ($2.96\% \pm 1.55\%$) (Fig. 2). Similarly, PCR revealed positive expression of k18 and p63 in these cells, while K13 was not expressed (Fig. 3). IHC confirmed these findings; homogeneous positive staining was observed for K18 and p63 (Fig. 4), while scant staining was noted for Nanog, K13 and K14 (Fig. 5). Following three weeks of culture of the K18+ /p63+-derived simple epithelial cells at the ALI, a multilayered epithelium emerged (Fig. 6). The constructed epithelium demonstrated a stratified,

FIG. 7. Immunohistochemistry revealed positive staining for K14 in the deep layers of the epithelium (A), K13 in the suprabasal layers (B), and e-cadherin in all layers of the epithelium (C) consistent with the vocal fold epithelium. An arrow indicates positive staining for laminin-5 in the interface between the epithelium and the gel demonstrating the presence of a basement membrane (D; 200- μ m scale bar for all images). Color images available online at www.liebertpub.com/tea

FIG. 8. Transmission electron microscopy confirmed the presence of a complete basement membrane (A; 2- μ m scale bar) and intercellular junctions (B; 50-nm scale bar), including tight junctions (TJ), adherens junctions (AJ), desmosomes (D), and gap junctions (G).

squamous vocal fold epithelial phenotype. Cells in the basal layer demonstrated positive staining for K14, a marker of the basal layer of stratified squamous epithelial cells (Fig. 7A). Cells in suprabasal layers were positive for K13, a marker of suprabasal cells in the vocal fold stratified squamous epithelium (Fig. 7B). While this pattern of staining was observed in each construct, we observed several discrete areas where suprabasal cells stained positive for K14.

Critical components of the epithelial barrier were observed in the constructs, including several types of intercellular junctions and basement membrane. Tight junctions were found at the apical aspects of adjacent cells, the most superficial layers of the constructed epithelium (Fig. 8B). Adherens junctions and desmosomes were observed in close proximity to the tight junctions. Desmosomes and gap junctions were observed in all layers of the epithelium. The presence of adherens junctions was confirmed with IHC using an anti-e-cadherin antibody (Fig. 7C). The basement membrane was present as evidenced by positive staining for laminin-5, an integral component of the basement membrane, under the basal layer (Fig. 7D). The presence of lamina lucida and lamina densa of the basement membrane was confirmed with electron microscopy (Fig. 8A).

The functional integrity of the epithelial barrier was evaluated by examining the ability of epithelial cells to inhibit penetration of a tracer, lanthanum nitrate, applied to the surface, into the epithelium. We observed lanthanum nitrate to penetrate to the most basal layer of the epithelium, indicating compromised epithelial barrier integrity (Fig. 9).

Discussion

In this study, we created an efficient, reproducible 3D model of vocal fold mucosa using ESC-derived simple epithelial cells and human primary vocal fold fibroblasts. The model is the first of its kind as it demonstrates key morphologic and phenotypic characteristics of native mucosa, including a multilayered, terminally differentiated epithelium and a continuous basement membrane. In the presence of an ALI, fibroblasts, and appropriate media, constructed epithelium demonstrated positive staining for the vocal fold stratified squamous epithelial markers K14 in the basal layer and terminally differentiated K13-positive cells in suprabasal layers. While this pattern of staining was not uniform

throughout all samples, there was a strong tendency for it to occur indicating that the model recapitulated cytokeratin distribution observed in native human vocal folds.

A critical role for the vocal fold epithelium is to provide a selective physical barrier between the airway and underlying lamina propria. The presence of intercellular junctions and basement membrane is a prerequisite for structural integrity. Here we show that the constructed mucosa demonstrated the presence of intercellular junctions and basement membrane, which provide the epithelium with this important feature. Tight junctions were observed primarily in the superficial layer, rather than in the deeper layers, of stratified squamous epithelial cells through electron microscopy. This is consistent with the distribution of tight junctions in the vocal fold epithelium.¹⁰ Adherens junctions and desmosomes were also observed consistent with their presence in humans.^{10–12} However, despite the presence of intercellular

FIG. 9. Permeation of lanthanum nitrate (arrow) to the basal layer of epithelial cells indicates that the engineered epithelium is leaky (2- μ m scale bar).

FIG. 10. Culture of K18+ /p63+-derived epithelial cells on a collagen I gel in the absence of fibroblasts yielded only sparse monolayers of cells on the gel surface (H&E stain; 100- μ m scale bar). Color images available online at www.liebertpub.com/tea

junctions, the epithelium was leaky as evidenced by permeation of the tracer, lanthanum nitrate, to the deepest layer of the epithelium. Thus, while tight junctions and adherens junctions are present in our model, they are not able to maintain the functional epithelial barrier integrity.

It is well accepted that paracrine signaling between epithelial cells and fibroblasts is critical for mucosal development *in vivo*. Here we confirmed that coculture of stem cell-derived simple epithelial cells with vocal fold fibroblasts is also critical for generating an epithelium *in vitro*. Exclusion of fibroblasts from the model yielded primarily monolayers

FIG. 11. Addition of a soluble factor, calcium (1 mM) to defined serum-free keratinocyte media (DSFM) and flavonoid adenine dinucleotide (FAD) media resulted in ciliation (arrows) of the superficial cell layers as confirmed by TEM (5- μ m scale bar).

of epithelial cells (Fig. 10) supporting findings reported elsewhere that the presence of both epithelial cells and fibroblasts is needed for the growth of a multilayered vocal fold epithelium.⁵ Our study also found that the origin of the fibroblasts seeded in the model dictates the phenotype of the derived epithelium. Inclusion of fibroblasts native to vocal folds was critical for developing the nonkeratinized stratified vocal fold epithelium. Coculture of hESC-derived epithelial progenitor cells with dermal fibroblasts has been shown previously to yield a keratinized, stratified epithelium characteristic of skin.¹⁵ Together, our findings indicate that the presence of fibroblasts is necessary for the development of a stratified, differentiated epithelium. Further, they suggest that the origin of fibroblasts influences the epithelial phenotype, consistent with observations that hESC-derived progenitors respond to organ-specific mesenchyme.¹⁹

Creating the appropriate microenvironment is critical for successful derivation of the epithelium. In the present study, we used the FAD medium as it provided optimal conditions for deriving a vocal fold-like epithelium. Use of DSFM yielded a multilayered epithelium with an undesirable weblike pattern as observed elsewhere.¹⁴ Addition of calcium (1 mM) to the medium to promote terminal differentiation of cells elicited ciliation of the superficial layer of the epithelium (Fig. 11). We discontinued use of calcium as vocal fold epithelium, unlike other respiratory epithelia, is not ciliated.

Adult and ESCs offer promising, abundant sources of cells for *in vitro* models. Use of stem cell-derived epithelial cells in our tissue construct overcomes the limitations of using primary cells, including restricted proliferative capacity in culture and heterogeneity of phenotypes. It will also reduce the need for animal trials and eliminate concerns regarding the limitations of translating findings from animal to human models. Additionally, it will allow a cost-effective study of vocal fold cells in a controlled environment in which, manipulations can be made in a manner that is not possible *in vivo*. Finally, substitution of patient-derived human-induced pluripotent stem cells for ESCs would permit modeling of the pathophysiology of patient-specific human disease in the vocal fold.

While ongoing work is needed to hone the culture microenvironment to improve barrier integrity of the construct, the epithelium recapitulates the key structural features in the native human vocal fold epithelium in very important ways. This innovative model described here is of human origin, demonstrates vocal fold epithelial morphology and phenotype, and has a complete, continuous basement membrane. No model developed previously demonstrates all of these features. Future applications of this model will include identifying and manipulating mechanisms underlying epithelial cells and fibroblasts, normal and aberrant crosstalk in response to daily irritants (e.g., cigarette smoke and acid reflux), and mechanical stress (e.g., vibration) to improve prevention and treatment of common voice disorders.

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Disclosure Statement

No competing financial interests exist.

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